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Production of Germline Chimeric Chickens, With High Transmission Rate of Donor-Derived Gametes, Produced by Transfer of Primordial Germ Cells

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ABSTRACT Germline chimeric chickens were produced by transfer of primordial germ cells from White Leghorn to Barred Plymouth Rock, and vice versa. Blood was collected from stage 13–15 embryos and primordial germ cells were concentrated by Ficoll density gradient centrifugation. Approximately 200 primordial germ cells were injected into the bloodstream through the dorsal aorta of stage 14–15 recipient embryos from which blood had been drawn via the dorsal aorta prior to the injection. Intact embryos were also prepared as recipients for White Leghorns only. The manipulated embryos were cultured in recipient eggshells until hatching. Germline chimerism of the chickens reaching maturity was examined by mating them with Barred Plymouth Rocks and donor-derived offspring were identified based on their feather color. The efficiency of production of germline chimeras was 95% (19/20). When primordial germ cells were transferred from White Leghorn to Barred Plymouth Rock, the average frequency of donor-derived offspring was 81% for three male chimeras (96% for one female chimera), and it was ~3.5 times higher for transfer in the opposite direction (23% for 6 male chimeras). Removing blood from recipient embryos prior to primordial germ cell injection enhanced the frequency of donor-derived offspring by 10% in resulting male chimeras. Male chimeras produced donor-derived offspring more frequently (~3.8 times) than female chimeras. Increases, decreases, or no changes were observed in the frequency of donor-derived offspring from the germline chimeras with increasing age. Male to female ratio of the offspring derived from the donor primordial germ cells did not deviate significantly in male and female chimeras, suggesting that primordial germ cells that have different sex from recipient embryos could not differentiate into functional gametes. The technique for primordial germ cell transfer employed in this experiment is simple to perform and resulted in the efficient production of germline chimeras with high transmission rates of donor-derived gametes. This system provides a powerful tool for avian embryo manipulation.

Key Words: Chick embryo, Germline chimerism, Transplantation

INTRODUCTION

Primordial germ cells (PGCs) are progenitors of ova and spermatozoa. Avian PGCs originate from the epiblast (Eyal-Giladi et al., 1981) and gradually move to the lower layer during the early stages of primitive streak formation (Sutasurya et al., 1983). They then appear in the hypoblast layer of the germinal crescent region (Swift, 1914; Clawson and Domm, 1969). Subsequently, they enter the developing blood vascular system and circulate temporarily throughout the embryo (Swift, 1914; Fujimoto et al., 1976). Finally, they migrate into the germinal ridge (Meyer, 1964; Kuwana, 1993) and differentiate into ova or spermatozoa.

Manipulation of the PGCs, such as by gene transfer, could ensure the transmission of genetic modifications to the next generation. Although the production of transgenic chickens has been attempted using several methods, e.g., retrovirus vector (Salter et al., 1986, 1987; Bosselman et al., 1989ab), sperm vector (Freeman and Bumstead, 1987; Gavora et al., 1991; Rottmann et al., 1992), and direct microinjection (Sang and Perry, 1989; Perry et al., 1991; Naito et al., 1991a, 1994; Love et al., 1994), these methods can only insert exogenous DNA into the host genome. The method using germline chimeras produced by transfer of PGCs or their progenitors can employ homologous recombination (Capecchi, 1989) for modifying a specific location within the genome. Germline chimeras have been produced by transfer of blastodermal cells (Petitte et al., 1990, 1993; Brazolot et al., 1991; Naito et al., 1991b, 1992; Watanabe et al., 1992; Carsience et al., 1993), but manipulation of PGCs contributes directly to the germline transmission. Various attempts have been made to produce germline chimeras by transfer of PGCs collected from germinal crescents (Reynaud, 1976; Wentworth et al., 1989; Vick et al., 1993ab) or embryonic

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blood (Simkiss et al., 1989; Petitte et al., 1991), and these attempts showed that the transferred PGCs differentiated normally into germ cells, giving rise to viable offspring (Reynaud, 1976; Wentworth et al., 1989; Vick et al., 1993a,b). However, the efficiency of obtaining donor-derived offspring in these chimeras was low, because the number of PGCs transferred to the recipient embryos was insufficient.

Recently, Yasuda et al. (1992) developed a technique for concentrating PGCs collected from the embryonic blood at stages 13–15 (Hamburger and Hamilton, 1951) by Ficoll density gradient centrifugation. Using this technique, Tajima et al. (1993b) succeeded in producing germline chimeras by injecting 100 PGCs isolated from the embryonic blood into the terminal sinuses of host embryos. Chicks derived from the donor PGCs, judged by their feather color, were obtained from the chimeras at an efficiency of up to 12%.

To enhance the efficiency of obtaining chicks derived from donor PGCs, it is necessary to eliminate endogenous PGCs from recipient embryos and to increase the number of PGCs for transfer. We have developed an efficient system for generating germline chimeric chickens with a high transmission rate of donor-derived gametes produced by transfer of PGCs isolated from early embryonic blood. These chimeric chickens can transmit genetically manipulated PGCs, prepared in vitro, to the germline.

MATERIALS AND METHODS

Fertilized eggs of White Leghorns (WL) and Barred Plymouth Rocks (BPR) were obtained from the genetic stocks maintained at the National Institute of Animal Industry. WL are homozygous dominant for pigment inhibitor gene (I/I), and BPR are homozygous recessive for the same locus (i/i). Transfer of PGCs was carried out from WL to BPR, and vice versa. The WL chickens injected with BPR PGCs at the embryonic stage and the BPR chickens injected with WL PGCs are referred to here as WL(BPR) and BPR(WL), respectively.

Counting Number of PGCs in the Bloodstream

The number of PGCs in the bloodstream was counted as follows. Blood (5 μl) collected from stage 15 embryos (Hamburger and Hamilton, 1951) of WL and BPR was diluted (to 200 μl) and fixed with 3.7% formaldehyde dissolved in phosphate-buffered saline. The samples (7 μl × 12) were placed on a glass cell culture slide and covered with 0.05% celloidin dissolved in ethanol (Tajima et al., 1993a). The fixed cells were then stained with periodic acid-Shiff (PAS) (Meyer, 1964) and PGCs (PAS-positive) were counted.

Preparation of Donor PGCs

Fertilized eggs were incubated at 38°C and 60% relative humidity in a forced air incubator (P-008, Showa Incubator Laboratory, Saitama, Japan) for ~53 hours to obtain embryos at stages 13–15 (Hamburger and Hamilton, 1951), when most PGCs circulate in the bloodstream (Singh and Meyer, 1967; Nakamura et al., 1988). Embryonic blood was collected from the dorsal aorta with fine glass micropipettes, which were made by pulling siliconized microcapillary tubing (inner diameter: 0.69 mm, outer diameter: 0.97 mm; Drummond). The tips were beveled down (25°) to an outer diameter of ~60 μm. The collected blood was pooled and dispersed in modified Hanks' solution supplemented with 10% fetal bovine serum (cHanks'; Yasuda et al., 1992). PGCs were then concentrated by Ficoll density gradient centrifugation (Yasuda et al., 1992). The cells collected from the PGC-rich fraction (the purity of PGCs in this fraction was ~60%; Fig. 1) were dispersed in 100 μl cHanks' solution and placed in a plastic dish (Falcon).

Preparation of Recipient Embryos

Recipient embryos were cultured in host eggshells to enable more precise manipulation. Fertilized eggs for recipient embryos were broken and the embryos (yolks) were transferred to small recipient eggshells, prepared from freshly laid eggs by drilling off the sharp end of the shell (33 mm in diameter of hole). The shells were filled with thin albumen collected from freshly laid eggs and sealed with cling film secured by plastic rings and elastic bands (Perry, 1988; Naito et al., 1990). The reconstituted eggs (recipient embryos) were incubated at 38°C for ~53 hours with rocking through an angle of 90° in every 5 minutes. When the embryo reached stage 14–15 (Hamburger and Hamilton, 1951), as observed through the aperture of the reconstituted egg, the plastic rings and cling film were taken off the reconstituted egg and the blood was drawn as much as possible (4–10 μl) from the dorsal aorta of the exposed embryo by a fine glass micropipette (~50 μm outer diameter). The egg was then resealed with cling film and plastic rings after adding a small amount of thin albumen to the embryo, and incubated at 38°C for 4–5 hours with rocking until injection of donor PGCs. Recipient embryos from which blood was not drawn were also prepared as for the reconstituted eggs, but for WL only.

Injection of PGCs Into Recipient Embryos

PGCs were readily distinguishable from blood cells by their remarkably large size and the presence of considerable numbers of refractive granules (lipids) in the cytoplasm as observed using phase contrast microscopy (Fujimoto et al., 1976). Approximately 200 PGCs were picked up by a fine glass micropipette (~40 μm outer diameter) from the collected cells in the plastic dish and dispersed in up to 1 μl cHanks' solution. The PGCs were injected into the bloodstream through the dorsal aorta of recipient embryos, which were exposed by taking off the cling film and plastic rings from the reconstituted egg. The manipulated embryos were then transferred to large recipient eggshells and incubated until hatching (Rowlett and Simkiss, 1987; Perry, 1988; Naito et al., 1990).

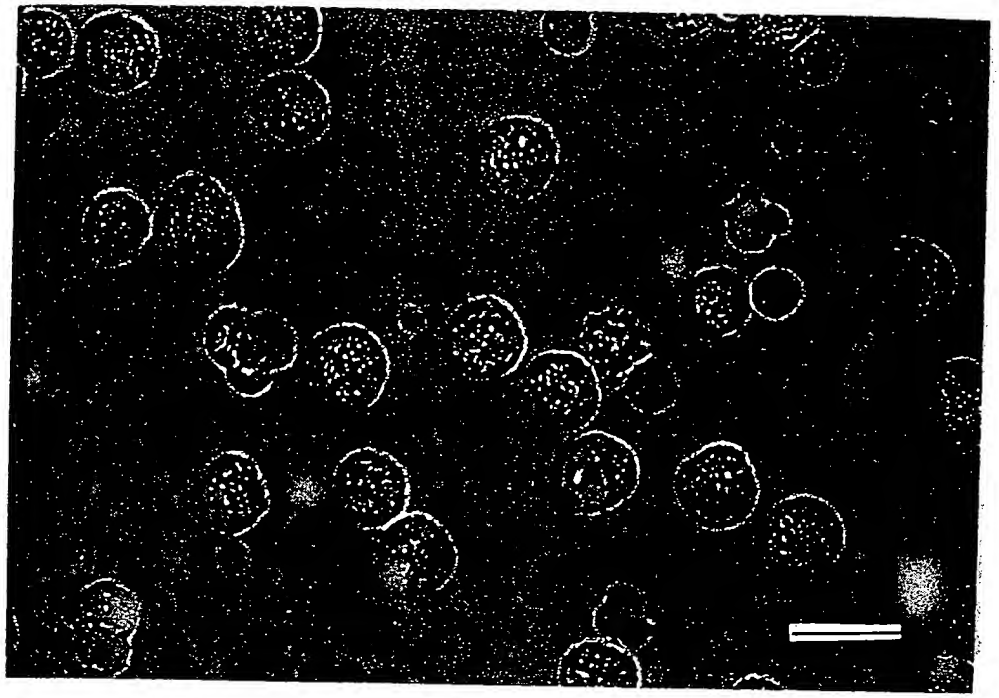

Fig. 1. PGCs collected from chick embryonic blood. Blood of stages 13–15 embryos was collected and PGCs (large cells with eccentrically placed nucleus and a considerable amount of lipids in the cytoplasm) were concentrated by Ficoll density gradient centrifugation. Small round cells are erythrocytes. Bar = 20 μm.

Frequency of Germline Transmission of Donor PGCs

WL(BPR) and BPR(WL) that survived to sexual maturity were mated with BPR by artificial insemination and the feather color of their offspring was examined. Black offspring (i/i) indicated that the offspring was derived from the donor PGC (BPR) when the parent was WL(BPR), and white offspring with small patches of black pigmentation (I/i) indicated that the offspring was derived from the donor PGC (WL) when the parent was BPR(WL). Sex of all the donor-derived offspring was determined by checking for the presence of testes or ovaries. Feather color and sex of the unhatched embryos were also determined, when possible, and included in the data. Some black offspring (BPR) derived from the donor PGCs were raised and examined to determine whether or not they had normal reproductive performance.

RESULTS

Numbers of PGCs in Bloodstream

The numbers of PGCs in 1 μl blood of stage 15 embryos (Hamburger and Hamilton, 1951) were 26.0 ± 7.8 and 45.1 ± 10.7 (mean ± s.d., n=10) in WL and BPR, respectively, and it was significantly ($P < 0.01$, F-test) different between the two breeds.

Survival and Hatching Rates of Manipulated Embryos

Drawing the blood and injection of PGCs through the dorsal aorta of recipient embryos could be carried out without hemorrhage. Survival and hatching rates of the chick embryos following injection of the PGCs into the embryonic bloodstream are shown in Table 1. The survival rates of the embryos from which blood was drawn prior to the PGC injection for both WL and BPR decreased mostly between 18 to 21 days compared with those of control embryos cultured in the same way. The rate of hatching showed only a slight decrease for manipulated embryos, suggesting that drawing the blood from the dorsal aorta of recipient embryos prior to PGC injection did not seriously affect embryonic development. The control embryos as well as manipulated embryos were transferred into large recipient eggshells at ~day 2.5 of incubation and the rate of hatching (~30%) was somewhat lower than when embryos were transferred at day 3 of incubation (~50%; Naito et al., 1990).

Germline Chimerism Obtained From Progeny Test

When WL PGCs were transferred to BPR recipients, male and female BPR(WL) tested were all germline chimeras (Table 2). The average frequency of germline transmission of donor PGCs was 81% in three male BPR(WL) (W-8226 is shown in Fig. 2) and 96% in one female BPR(WL). When BPR PGCs were transferred to WL recipients, 10 male and 5 female WL(BPR) tested were germline chimeras and one female WL(BPR) was not (Tables 3, 4). When the blood was drawn from the recipient embryos prior to the PGC injection, the average frequency of germline transmission of donor PGCs was 23% in six male WL(BPR) and 6% in four female

TABLE 1. Survival and Hatching Rates of Chick Embryos Following Injection of PGCs Into the Embryonic Bloodstream

Embryos	Number of embryos manipulated	Number (%) of embryos surviving on days[a] 3	7	14	18	21	Hatch (%)
BPR(WL)[b]	39	39 (100.0)	36 (92.3)	32 (82.1)	25 (64.1)	11 (28.2)	11 (28.2)
BPR[d]	22	22 (100.0)	19 (86.4)	16 (72.7)	11 (50.0)	7 (31.8)	7 (31.8)
WL(BPR)[b]	78	78 (100.0)	67 (85.9)	61 (78.2)	43 (55.1)	15 (19.2)	14 (17.9)
WL(BPR)[c]	28	28 (100.0)	19 (67.9)	18 (64.3)	15 (53.6)	7 (25.0)	7 (25.0)
WL[d]	100	100 (100.0)	95 (95.0)	82 (82.0)	62 (62.0)	34 (34.0)	30 (30.0)

[a]Days of incubation.
[b]Blood was drawn from the recipient embryos prior to PGC injection.
[c]PGCs were injected into the intact embryos.
[d]Control embryos were cultured in recipient eggshells.
Donor embryos from which PGCs were collected are shown in parentheses. WL: White Leghorn, BPR: Barred Plymouth Rock.

TABLE 2. Progeny Test of Germline Chimeras Produced by Transfer of PGCs From WL to BPR

	No.	Test period (weeks)	No. of chicks hatched	No. of white chicks	No. of black chicks	White chicks (%)	Sex ratio of white chicks[a] (♂:♀)
Male[b]	W-8224	9	70	57	13	81.4	26:29
	W-8226	68	820	631	189	77.3	313:293
	W-8229	8	68	57	11	83.8	24:32
Female[b]	W-8231	64	239	229	10	95.8	118:98

[a]Unhatched embryos whose sex was unknown are excluded.
[b]Blood was drawn from the recipient embryos prior to PGC injection.

WL(BPR) (Table 3). When PGCs were injected into the intact recipient embryos, the average frequency of germline transmission of donor PGCs was 14% in four male WL(BPR) and 62% in one female WL(BPR) (Table 4).

The average percentage of donor-derived offspring for male BPR(WL) (81%) for which blood was drawn from the recipient embryos prior to the PGC injection was significantly ($P < 0.01$, χ^2 test) higher than that for male WL(BPR) (23%) for which manipulation was conducted in the same way (Tables 2, 3). Also, the average percentage of donor-derived offspring for male WL(BPR) (23%) for which blood was drawn from the recipient embryos prior to the PGC injection was significantly ($P < 0.01$) higher than that for male WL(BPR) (14%) for which PGCs were injected into the intact recipient embryos (Tables 3,4). Comparing the male and female chimeras, the average percentage of donor-derived offspring for male WL(BPR) (23%) was significantly ($P < 0.01$) higher than that for female WL(BPR) (6%) (Table 3). But in the female WL(BPR), W-8243, and the female BPR(WL), W-8231, more than half of the offspring were derived from the donor PGCs.

Offspring (BPR) derived from the donor PGCs reached maturity and 92.3% (84/91) for both fertility and hatchability were obtained.

Changes in Frequency of Donor-Derived Offspring

Changes in the frequency of donor-derived offspring produced by BPR(WL) and WL(BPR) with increasing age, estimated in 4-week periods, are shown in Table 5 (male) and Table 6 (female). In most of the BPR(WL) and WL(BPR) no apparent change was observed in the frequency of donor-derived offspring throughout the test period (a maximum of 68 weeks), whereas the frequency gradually increased significantly in one female WL(BPR) and gradually decreased significantly in four male WL(BPR) (Tables 5, 6; regression analysis).

Male-to-Female Ratio of Donor-Derived Offspring

The ratio of male:female offspring derived from donor PGCs for male BPR(WL) and male WL(BPR) was 743:737 and was 218:221 for female BPR(WL) and female WL(BPR). These ratios are not significantly different (χ^2 test) from 1:1.

DISCUSSION

In this study we report the production of germline chimeras by transfer of PGCs at a very high efficiency

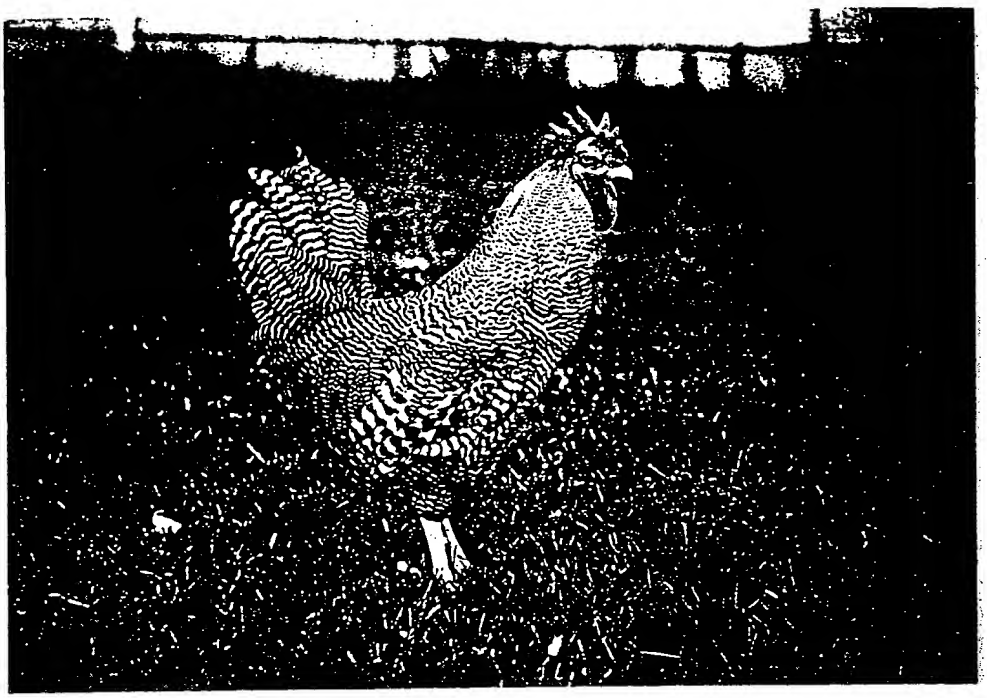

Fig. 2. Germline chimeric chicken (male, W-8226) produced by transfer of approximately 200 WL PGCs into BPR recipient embryo from which blood (6 μl) had been drawn prior to the injection. This chimeric chicken produced 631 donor-derived offspring during a 68-week test period by mating with three BPR females. The extent of germline transmission of donor PGCs (WL) was 77.3% (Tables 2, 5).

TABLE 3. Progeny Test of Germline Chimeras Produced by Transfer of PGCs from BPR to WL

	No.	Test period (weeks)	No. of chicks hatched	No. of white chicks	No. of black chicks	Black chicks (%)	Sex ratio of black chicks[a] (♂:♀)
Male[b]	W-8237	25	255	205	50	19.6	22:24
	W-8238	16	180	125	55	30.6	24:24
	W-8240	42	376	296	80	21.3	42:35
	W-8245	56	644	346	298	46.3	143:126
	W-8246	37	483	436	47	9.7	22:21
	W-8250	32	404	355	49	12.1	24:23
Female[b]	W-8236	49	268	268	0	0.0	
	W-8241	51	199	195	4	2.0	3:1
	W-8244	51	284	252	32	11.3	10:22
	W-8248	48	282	256	26	9.2	11:15
	W-8251	48	240	235	5	2.1	2:3

[a]Unhatched embryos whose sex was unknown are excluded.
[b]Blood was drawn from the recipient embryos prior to PGC injection.

of 95% (19/20). In avian species, PGCs circulate temporarily in the developing bloodstream, and this unique migratory pathway makes PGC transfer simple compared with that in other species (Nieuwkoop and Sutasurya, 1979). PGCs injected into the bloodstream can be incorporated into the germinal epithelium within 24 hours after injection (Yasuda et al., 1992) and give rise to viable offspring (Tajima et al., 1993b). Injection of PGCs into the terminal sinus of the recipient embryo and the insertion of bubbles to prevent hemorrhages (Yasuda et al., 1992; Tajima et al., 1993b) requires a highly refined technique. The method of injecting PGCs into the dorsal aorta of recipient embryos that is reported here is simple to perform with no treatment needed to prevent hemorrhages, yet results in the efficient production of germline chimeras with high rates of transmission of donor-derived gametes.

The frequency of germline transmission of donor PGCs reported in this study was significantly different between male BPR (WL) and male WL(BPR). When WL PGCs were transferred into BPR recipients, most of the offspring obtained from male BPR(WL) were d rived from the donor PGCs with an average frequency (81%) ~3.5 times higher than for the opposite combination

TABLE 4. Progeny Test of Germline Chimeras Produced by Transfer of PGCs From BPR to WL (intact embryos)

	No.	Test period (weeks)	No. of chicks hatched	No. of white chicks	No. of black chicks	Black chicks (%)	Sex ratio of black chicks[a] (♂:♀)
Male[b]	W-8233	50	588	502	86	14.6	35:48
	W-8234	50	508	429	79	15.6	36:40
	W-8239	42	308	248	60	19.5	27:30
	W-8249	32	307	290	17	5.5	5:12
Female[b]	W-8243	49	260	98	162	62.3	74:82

[a]Unhatched embryos whose sex was unknown are excluded.
[b]PGCs were injected into the intact embryos.

(male WL(BPR); 23%). The number of PGCs in the bloodstream of stage 15 embryos (Hamburger and Hamilton, 1951) was ~1.7 times higher in BPR compared with that in WL, suggesting that drawing blood from recipient embryos prior to PGC injection might be more effective for BPR than for WL. Perhaps the apparent dominance of WL over BPR in the competition of germ cell proliferation in the recipient gonads reflects the difference in the egg laying performance between WL and BPR. Further studies are required, however, on the difference in frequency of germline transmission of donor PGCs for BPR(WL) and WL(BPR).

Removing the blood from the recipient embryos prior to PGC injection enhanced the frequency of donor-derived offspring from 14 to 23% for male WL(BPR). Several methods have been attempted to eliminate or reduce endogenous PGCs of recipient embryos: ultraviolet irradiation (Reynaud, 1976; Aige-Gil and Simkiss, 1991a), laser irradiation (Mims and McKinnell, 1971), application of busulfan (Eige-Gil and Simkiss, 1991b; Hallett and Wentworth, 1991; Vick et al., 1993b), application of concanavalin A (Al-Thani and Simkiss, 1991), excision of germinal crescent region (McCarrey and Abbott, 1982). But these methods also affect embryonic development and donor PGCs. Carsience et al. (1993) impaired the development of recipient embryos (stage-X blastoderms; Eyal-Giladi and Kochav, 1976) by exposure to γ radiation prior to the injection of blastodermal cells. The frequency of germline transmission of the donor blastodermal cells in the germline chimeras was 1.3–100% (median rate was 5.8%), whereas it was <0.4% when the recipient embryos were not irradiated (Carsience et al., 1993; Petitte et al., 1990, 1993). Removing blood from the dorsal aorta of recipient embryos prior to PGC injection, as reported here, seems to be the most effective of the presently applicable methods for the production of germline chimeras, since it is simple to perform and has less impact on embryonic development. If embryonic blood could be completely removed from recipient embryos at the peak of PGC circulation in the bloodstream, the frequency of germline transmission of donor PGCs could be further enhanced.

Increasing, decreasing, and unchanged frequencies of donor-derived offspring from germline chimeras were observed with increasing age. Similar tendencies were observed for WL and Rhode Island Red (RIR) reciprocal produced by Tajima et al. (1993b). These patterns did not relate to the initial level of production of donor-derived offspring or whether or not blood was removed prior to the PGC injection. In the four male WL(BPR) showing the decreasing pattern of production of donor-derived offspring, the percentage of BPR sperms might have decreased with increasing age because the activity of BPR spermatogonia was different from that of WL spermatogonia.

The frequency of donor-derived offspring of male WL(BPR) was much higher than of female WL(BPR), although it was unexpectedly high in W-8243, a female WL(BPR). The same tendency was observed in the germline chimeras produced by blastodermal cell transfer from BPR to WL (Carsience et al., 1993). However, this sex difference was not obvious in the germline chimeras, WL(RIR) and RIR(WL) (Tajima et al., 1993b). In the present experiment the donor PGCs were a mixed population of cells bearing ZZ and ZW chromosomes. If PGCs bearing ZZ chromosomes injected into female recipients can give rise to viable offspring, the expected male to female ratio of offspring is 3:1, and if PGCs bearing ZW chromosomes injected into male recipients can give rise to viable offspring, the expected male to female ratio of offspring is 3:4. The results of the present experiment show that the male-to-female ratio of donor-derived offspring produced by both male and female chimeras did not deviate significantly from 1:1. This equal ratio of male and female offspring from the male and female chimeras suggests that the donor PGCs that have different sex from the recipient embryos could not differentiate into functional gametes. Therefore, donor PGCs bearing ZW chromosomes could not differentiate into functional sperms with W chromosome in male chimeras. Shaw et al. (1992) reported that female cells were usually excluded from adult male chimeras produced by blastodermal cell transfer. Thus the cause of this difference in the frequency of donor-derived offspring in male and female chimeras is unknown, and further studies are required to clarify this issue.

Germline chimeras are potentially very useful for avian embryo manipulation, especially for gene transfer. Although direct microinjection of exogenous DNA into the germinal disc of fertilized chick ova has become

TABLE 5. Changes in Percentage of Donor-Derived Offspring Produced by Male BPR(WL) and WL(BPR)

4-week period	BPR(WL)[a]			WL(BPR)[a]						WL(BPR)[b]			
	W-8224	W-8226	W-8229	W-8237	W-8238	W-8240	W-8245	W-8246	W-8250	W-8233	W-8234	W-8239	W-8249
1	85.2	76.6	89.7	22.2	26.9	54.8	38.1	12.1	11.9	20.4	49.1	17.0	10.8
2	78.6	88.5	79.5	23.9	35.8	21.4	46.4	8.3	6.3	13.6	31.5	42.6	8.3
3		83.6		22.7	13.6	33.3	52.8	8.6	11.1	7.3	4.7	20.0	5.4
4		71.9		14.5	35.8	25.0	50.0	12.2	15.2	10.0	7.0	24.0	3.6
5		72.7		24.2		15.4	52.6	12.2	20.8	14.0	14.6	—	5.4
6		70.8		12.5		23.3	53.3	5.9	10.6	16.3	13.6	21.4	4.8
7		81.4				12.5	46.7	8.5	14.6	14.9	13.9	13.0	2.6
8		65.0				11.4	45.2	8.9	8.7	14.0	3.1	9.1	2.6
9		83.3				4.9	55.6	12.3		25.8	3.0	9.1	
10		65.9				12.5	47.4			15.8	16.3	8.0	
11		81.3					35.3			19.1	6.5		
12		85.4					39.2			13.0	4.0		
13		77.0					43.4						
14		80.0					47.1						
15		74.1											
16		74.5											
17		76.4											
b[c]		−0.13		−1.59	0.45	−3.85**	−0.36	−0.07	0.28	0.37	−2.43*	−2.43*	−1.02**

[a]Blood was drawn from the recipient embryos prior to PGC injection. Donor embryos from which PGCs were collected are shown in parentheses.
[b]PGCs were injected into the intact embryos.
[c]Regression coefficient of the percentage of donor-derived offspring during the 4-week period (**$P < 0.01$, *$P < 0.05$).

TABLE 6. Changes in Percentage of Donor-Derived Offspring Produced by Female BPR(WL) and WL(BPR)

4-week period	BPR(WL)[a]	WL(BPR)[a]					WL(BPR)[b]
	W-8231	W-8236	W-8241	W-8244	W-8248	W-8251	W-8249
1	78.6	0.0	0.0	0.0	4.5	0.0	43.8
2	100.0	0.0	0.0	4.0	22.2	5.6	50.0
3	94.7	0.0	0.0	8.0	11.1	0.0	60.7
4	100.0	0.0	4.3	11.1	16.0	4.0	69.2
5	—	0.0	0.0	5.0	11.5	7.7	76.2
6	—	0.0	4.8	0.0	8.0	0.0	55.6
7	100.0	0.0	0.0	16.0	3.7	0.0	60.0
8	95.0	0.0	0.0	3.8	4.8	0.0	54.5
9	100.0	0.0	0.0	15.0	8.3	0.0	89.5
10	95.2	0.0	12.5	9.5	4.3	0.0	66.7
11	95.0	0.0	0.0	29.2	4.2	5.3	71.4
12	87.5	0.0	0.0	20.8	15.0	0.0	50.0
13	100.0		0.0	21.4			
14	88.2						
15	100.0						
16	100.0						
b[c]	0.29	0.00	0.11	1.74**	−0.55	−0.16	1.20

[a]Blood was drawn from the recipient embryos prior to PGC injection. Donor embryos from which PGCs were collected are shown in parentheses.
[b]PGCs were injected into the intact embryos.
[c]Regression coefficient of the percentage of donor-derived offspring during the 4-week period (**$P < 0.01$).

possible recently (Love et al., 1994), the efficiency of introduction of exogenous DNA into somatic and germ cells of chickens is low (0.76%; Naito et al., 1994). Transgenic chickens can be produced from germline chimeras produced by transfer of PGCs transfected with retrovirus vectors (Vick et al., 1993). In the germline chimeras produced by injecting WL PGCs into BPR recipients, reported here, the average frequency of germline transmission of the donor PGCs in male and female BPR(WL) was 85%. By combining this technique with efficient transfection of PGCs, transgenic chickens can be produced very efficiently. Moreover, specific gene targeting may be possible by selection in vitro for the desired recombinant using cultured PGCs, allowing molecular approaches to be applied to understanding various biological phenomena in avian species. Also, cryopreservation of PGCs can be applied to preserve genetic material, and we have obtained successful results in this area (Naito et al., unpublished results).

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REFERENCES

Aige-Gil V, Simkiss K (1991a): Sterilising embryos for transgenic chimaeras. Brit Poult Sci 32:427–438.

Aige-Gil V, Simkiss K (1991b): Sterilisation of avian embryos with busulphan. Res Vet Sci 50:139–144.

Al-Thani R, Simkiss K (1991): Effects of an acute in vivo application of concanavalin A on the migration of avian germ cells. Protoplasma 161:52–57.

Bosselman RA, Hsu R-Y, Boggs T, Hu S, Bruszewski J, Ou S, Kozar L, Martin F, Green C, Jacobsen F, Nicolson M, Schultz JA, Semon KM, Rishell W, Stewart RG (1989a): Germline transmission of exogenous genes in the chicken. Science 243:533–535.

Bosselman RA, Hsu R-Y, Boggs T, Hu S, Bruszewski J, Ou S, Souza L, Kozar L, Martin F, Nicolson M, Rishell W, Schultz JA, Semon KM, Stewart RG (1989b): Replication-defective vectors of reticuloendotheliosis virus transduce exogenous genes into somatic stem cells of the unincubated chicken embryo. J Virol 63:2680–2689.

Brazolot CL, Petitte JN, Etches RJ, Verrinder Gibbins AM (1991): Efficient transfection of chicken cells by lipofection, and introduction of transfected blastodermal cells into the embryo. Mol Reprod Dev 30:304–312.

Capecchi MR (1989): Altering the genome by homologous recombination. Science 244:1288–1292.

Carsience RS, Clark ME, Verrinder Gibbins AM, Etches RJ (1993): Germline chimeric chickens from dispersed donor blastodermal cells and compromised recipient embryos. Development 117:669–675.

Clawson RC, Domm LV (1969): Origin and early migration of primordial germ cells in the chick embryo: A study of the stages definitive primitive streak through eight somites. Am J Anat 125:87–112.

Eyal-Giladi H, Kochav S (1976): From cleavage to primitive streak formation: A complementary normal table and a new look at the first stages of the development of the chick I. General Morphology. Dev Biol 49:321–337.

Eyal-Giladi H, Ginsburg M, Farbarof A (1981): Avian primordial germ cells are of epiblastic origin. J Embryol exp Morph 65:139–147.

Freeman BM, Bumstead N (1987): Transgenic poultry: Theory and practice. World's Poult Sci J 43:180–189.

Fujimoto T, Ninomiya T, Ukeshima A (1976): Observations of the primordial germ cells in blood samples from the chick embryo. Dev Biol 49:278–282.

Gavora JS, Benkel B, Sasada H, Cantwell WJ, Fiser P, Teather RM, Nagai J, Sabour, M.P. (1991): An attempt at sperm-mediated gene transfer in mice and chickens. Can J Anim Sci 71:287–291.

Hallett JS, Wentworth BC (1991): The effects of busulfan on gonadal

differentiation and development in Japanese quail *(Coturnix coturnix japonica)*. Poult Sci 70:1619–1623.

Hamburger V, Hamilton HL (1951): A series of normal stages in the development of the chick embryo. J Morph 8:49–92.

Kuwana T (1993): Migration of avian primordial germ cells toward the gonadal anlage. Dev Growth Differ 35:237–243.

Love J, Gribbin C, Mather C, Sang H (1994): Transgenic birds by DNA microinjection. Bio/technol 12:60–63.

McCarrey JR, Abbott UK (1982): Functional differentiation of chick gonads following depletion of primordial germ cells. J Embryol exp Morph 68:161–174.

Meyer DB (1964): The migration of primordial germ cells in the chick embryo. Dev Biol 10:154–190.

Mims MF, McKinnel RG (1971): Laser irradiation of the chick embryo germinal crescent. J Embryol exp Morph 26:31–36.

Naito M, Nirasawa K, Oishi T (1990): Development in culture of the chick embryo from fertilized ovum to hatching. J Exp Zool 254:322–326.

Naito M, Nirasawa K, Oishi T (1992): Preservation of quail blastoderm cells in liquid nitrogen. Brit Poult Sci 33:449–453.

Naito M, Sasaki, E, Ohtaki M, Sakurai M (1994): Introduction of exogenous DNA into somatic and germ cells of chickens by microinjection into the germinal disc of fertilized ova. Mol Reprod Dev 37:167–171.

Naito M, Watanabe M, Kinutani M, Nirasawa K, Oishi T (1991b): Production of quail-chick chimaeras by blastoderm cell transfer. Brit Poult Sci 32:79–86.

Naito M, Agata K, Otsuka K, Kino K, Ohta M, Horose K, Perry MM, Eguchi G (1991a): Embryonic expression of β-actin-lacZ hybrid gene injected into the fertilized ovum of the domestic fowl. Int J Dev Biol 35:69–75.

Nakamura M, Kuwana T, Miyayama Y, Fujimoto T (1988): Extragonadal distribution of primordial germ cells in the early chick embryo. Anat Rec 222:90–94.

Nieuwkoop PD, Sutasurya LA (1979): "Primordial Germ Cells in the Chordates." Cambridge: Cambridge University Press.

Perry MM (1988): A complete culture system for the chick embryo. Nature 331:70–72.

Perry MM, Morrice D, Hettle S, Sang H (1991): Expression of exogenous DNA during the early development of the chick embryo. Roux's Arch Dev Biol 200:312–319.

Petitte JN, Brazolot CL, Clark ME, Liu G, Verrinder Gibbins AM, Etches RJ (1993): Accessing the genome of the chicken using germline chimeras. In RJ Etches, AM Verrinder Gibbins (ed): "Manipulation of the Avian Genome." Boca Raton: CRC Press, pp 81–101.

Petitte JN, Clark ME, Etches RJ (1991): Assessment of functional gametes in chickens after transfer of primordial germ cells. J Reprod Fert 92:225–229.

Petitte JN, Clark ME, Liu G, Verrinder Gibbins AM, Etches RJ (1990): Production of somatic and germline chimeras in the chicken by transfer of early blastodermal cells. Development 108:185–189.

Reynaud G (1976): Reproductive capacity and offspring of chickens submitted to a transfer of primordial germ cells during embryonic life. Roux's Arch Dev Biol 179:85–110.

Rottmann OJ, Antes R, Hofer P, Maierhofer G (1992): Liposome mediated gene transfer via spermatozoa into avian egg cells. J Anim Breed Genet 109:64–70.

Rowlett K, Simkiss K (1987): Explanted embryo culture: In vitro and in ovo techniques for domestic fowl. Brit Poult Sci 28:91–101.

Salter DW, Smith EJ, Hughes SH, Wright SE, Crittenden LB (1987): Transgenic chickens: Insertions of retroviral genes into the chicken germ line. Virology 157:236–240.

Salter DW, Smith EJ, Hughes SH, Wright SE, Fadly AM, Witter RL, Crittenden LB (1986): Gene insertion into the chicken germ line by retroviruses. Poult Sci: 65:1445–1458.

Sang H, Perry MM (1989): Episomal replication of cloned DNA injected into the fertilized ovum of the hen, *Gallus domesticus*. Mol Reprod Dev 1:98–106.

Shaw DL, Carsience RS, Etches RJ, Verrinder Gibbins AM (1992): The fate of female donor blastodermal cells in male chimeric chickens. Biochem Cell Biol 70:1218–1229.

Simkiss K, Rowlett K, Bumstead N, Freeman BM (1989): Transfer of primordial germ cell DNA between embryos. Protoplasma 151:164–166.

Singh RP, Meyer DB (1967): Primordial germ cells in blood smears from chick embryos. Science 156:1503–1504.

Sutasurya LA, Yasugi S, Mizuno T (1983): Appearance of primordial germ cells in young chick blastoderms cultured in vitro. Dev Growth Differ 25:517–521.

Swift CH (1914): Origin and early history of the primordial germ cells in the chick. Am J Anat 15:483–516.

Tajima A, Kamisumi A, Yasuda Y, Chikamune T (1993a): A study on the number of primordial germ cells in the blood stream of early chick embryos. Proc 87th Annual Meeting Jpn Soc Zootech Sci IX: 307.

Tajima A, Naito M, Yasuda Y, Kuwana T (1993b): Production of germ line chimera by transfer of primordial germ cells in domestic chicken *(Gallus domesticus)*. Theriogenology 40:509–519.

Vick L, Li Y, Simkiss K (1993a): Transgenic birds from transformed primordial germ cells. Proc R Soc Lond B 251:179–182.

Vick L, Luke G, Simkiss K (1993b): Germ-line chimaeras can produce both strains of fowls with high efficiency after partial sterilization. J Reprod Fert 98:637–641.

Watanabe M, Kinutani M, Naito M, Ochi O, Takashima Y (1992): Distribution analysis of transferred donor cells in avian blastodermal chimeras. Development 114:331–338.

Wentworth BC, Tsai H, Hallett JH, Gonzales DS, Rajcic-Spasojevic G (1989): Manipulation of avian primordial germ cells and gonadal differentiation. Poult Sci 68:999–1010.

Yasuda Y, Tajima A, Fujimoto T, Kuwana T (1992): A method to obtain avian germ-line chimaeras using isolated primordial germ cells. J Reprod Fert 96:521–528.